Thromboxane hypersensitivity in hypoxic pulmonary artery myocytes: altered TP receptor localization and kinetics

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Hinton M, Gutsol A, Dakshinamurti S. Thromboxane hypersensitivity in hypoxic pulmonary artery myocytes: altered TP receptor localization and kinetics. Am J Physiol Lung Cell Mol Physiol 292: L654–L663, 2007. First published November 3, 2006; doi:10.1152/ajplung.00229.2006.—Hypoxia-induced neonatal persistent pulmonary hypertension (PPHN) is characterized by sustained vasospasm and increased thromboxane (TxA2)-to-prostacyclin ratio. We previously demonstrated that moderate hypoxia induces myocyte TxA2 hypersensitivity. Here, we examined TxA2 prostanooid receptor (TP-R) localization and kinetics following hypoxia to determine the mechanism of hypoxia-induced TxA2 hypersensitivity. Primary cultured neonatal pulmonary artery myocytes were exposed to 10% O2 (hypoxic myocytes; HM) or 21% O2 (normoxic myocytes; NM) for 3 days. PPHN was induced in neonatal piglets by in vivo exposure to 10% FIO2 for 3 days. TP-R was studied in whole lung sections from pigs with hypoxic PPHN and age-matched controls; intracellular localization was studied by immunocytochemistry. TP-R affinity was studied in cultured myocytes by saturation binding kinetics using3H-SQ-29548 and competitive binding kinetics by coinubcation with U-46619. Phosphorylation and coupling were examined in immunoprecipitated TP-R. We report distal propagation of TP-R expression in PPHN, extending to pulmonary arteries <50 μm. In HM, intracellular TP-R moves towards the perinuclear region, mirroring a change in endoplasmic reticulum (ER) morphology. TP-R kinetics also alter in HM membranes, with decreased k4 and Bmax (maximal binding sites). Additionally, in hypoxia,3H-SQ-29548 is displaced at lower concentration of U-46619 than in normoxia, suggesting increased agonist affinity. Phosphorylation of serine residues on HM TP-R was significantly decreased compared with NM; this difference correlated with increased Goa coupling in hypoxia and was ablated by incubation with PKA. We conclude that the TP-R is normally desensitized in the neonatal pulmonary circuity by PKA-mediated regulatory phosphorylation, decreasing ligand affinity and coupling to Goa; this protection is lost following hypoxic exposure. Also, the appearance of TP-R in resistance arteries after development of hypoxic PPHN may contribute to increased pulmonary arterial pressure.

smooth muscle; persistent pulmonary hypertension of the newborn; Scatchard analysis

AT BIRTH, THE PULMONARY CIRCUIT must reduce its high vascular resistance to accommodate an eight-to-ten-fold increase in blood flow. This transition requires pulmonary inflation with oxygen and active vasodilation by nitric oxide (NO) and prostacyclin (PGI2) (43). One of the most rapidly progressive and potentially fatal of the vasculopathies, neonatal persistent pulmonary hypertension (PPHN) has an incidence of up to 6.8 in 1,000 live births (52). PPHN is caused in otherwise healthy term infants by interference of normal circulatory transition by perinatal hypoxia, inflammation, or direct lung injury, such as meconium aspiration (18), and is characterized by sustained vasospasm and chronic vascular remodeling (50). All etiologies of PPHN result in a critical decrease in tissue oxygen delivery (15). Approximately one-third of patients meeting treatment criteria do not respond to therapeutic agents, including inhaled NO, and in this subgroup the disease is lethal, although rescue therapy with extracorporeal membranous oxygenation may limit mortality (9).

Hypoxic pulmonary vasoconstriction may be physiologically advantageous to bypass localized hypoventilated areas of lung. However, chronic hypoxia is a proinflammatory stimulus that induces both proliferation (56) and constriction (39) in vascular smooth muscle. It is known that alveolar hypoxia (10% O2) rapidly induces macrophage recruitment, increases vascular permeability, and enhances expression of inflammatory mediators TNF-α, NF-κB, ICAM-1, and macrophage inflammatory protein-1β (MIP-1β), as well as hypoxia-inducible factor-1α (HIF-1α) (28). Hypoxia also worsens lipopolysaccharide-induced injury in rat lung; the hypoxia-induced component of the inflammatory response is independently generated and localized to the respiratory compartment (49). Cyclooxygenase-2 (COX-2) upregulation in hypoxia is described in many species (27), as well as in human pulmonary arterial myocytes (58). Altered arachidonic acid metabolism with an increased thromboxane (TxA2)-to-PGI2 ratio is described early in the course of PPHN, in the neonatal piglet hypoxia model (6); this shift toward the inflammatory arachidonic acid metabolites mediates increased pulmonary vasoconstriction. Hypoxia has a priming effect on pulmonary vascular smooth muscle agonist response and increases inositol triphosphate (IP3) generation to agonist, favoring myocyte contraction (36). Long-term hypoxia can result in chronic vasospasm by decreasing K+ channel open probability and downregulating Kv channel expression (32). Although multiple downstream effects of hypoxia have been demonstrated, the intracellular sensor of O2 tension is unknown but may include ROS production and/or heme-containing proteins (20).

The major endogenous molecules that regulate pulmonary vascular tone and are pivotal in the perinatal period include the NO-endothelin and PGI2-TxA2 axes (54). A shift in the NO-endothelin ratio away from production of the vasorelaxant NO, due to decreased endothelial NO synthase expression, has been shown to contribute to the development of PPHN (48). In
addition, an increased TxA₂/PGI₂ ratio, due to decreased PGI₂ synthase production, has been described in a hypoxic model of PPHN (6). TxA₂ is a constrictor prostanooid, produced via the arachidonic acid pathway in response to oxidative stress and proinflammatory stimuli, and is known to be crucial in mediating septic pulmonary hypertension in the neonate (5, 13). COX pathway metabolites are implicated in increased pulmonary vascular tone, contributing to the early pulmonary hyperensive response in meconium aspiration (41) and sepsis (13).

We have previously shown that neonatal pulmonary artery myocytes exposed to a moderate level of hypoxia have hypersensitive and hyperresponsive peak [Ca²⁺], responses to the TxA₂ agonist U-46619, despite a reduction in cell surface TxA₂ prostaglandin receptor (TP-R) expression (19); this heightened response persists long after removal from hypoxia.

TP-R binds to the TP-R, which is a member of the G protein-coupled receptor (GPCR) superfamily (23). Differential splicing of the TP-R COOH-terminal tail gives rise to the protein-coupled receptor (GPCR) superfamily (23). Differented response persists long after removal from hypoxia.

TP-R alters its activity state, with phosphorylation leading to number and affinity in platelet membranes (4) and transfected TP-R couple to Gₐ but alternatively regulate adenylate cyclase through activation by TP-Rα or inhibition by TP-Rβ (21). Signaling through Goₐ leads to activation of phospholipase C, which produces diacylglycerol and IP₃ (33). Altered hypoxia in vivo and in vitro and detailed receptor-ligand interaction (42).

The left shift in the TxA₂ dose-response curve observed following moderate hypoxic exposure suggests a probable change in TP-R kinetics, involving alteration of either receptor abundance or affinity. Whereas GPCRs are most commonly upregulated by increasing receptor abundance, the mechanism of hypoxia-induced TxA₂ hypersensitivity in the neonatal pulmonary circuit has not been previously studied. In this study, we examine TP-R localization following exposure to moderate hypoxia in vivo and in vitro and detailed receptor-ligand kinetics in myocytes after in vitro hypoxia. We hypothesize that the hypersensitive TxA₂ response observed following moderate hypoxic exposure is due to an increased affinity of the TP-R for agonist and that in vivo TP-R localization in the pulmonary arterial circuit is altered following development of PPHN.

METHODS

Animal model and induction of PPHN. All primary cell cultures were derived from newborn piglets (<24 h old; n = 13) that were killed on the day of arrival from a pathogen-free farm supplier. Lung tissue for histological analysis was obtained from pigs with hypoxia-induced PPHN (n = 4) or from age-matched controls (n = 4). This study protocol was reviewed and approved by an institutional review board following Canadian Council on Animal Care guidelines. The in vivo hypoxic model has been previously described (8). Briefly, newborn piglets were placed in a normobaric hypoxic chamber (FiO₂, 0.10, achieved by a mixture of room air with N₂) for 3 days. All piglets were euthanized by pentobarbital overdose and exsanguina.

Heart and lungs were removed en bloc and placed in oxygenated, cold (4°C) Ca²⁺-free Krebs-Henseleit physiological buffer (containing in mM: 112.6 NaCl, 25 NaHCO₃, 1.38 NaH₂PO₄, 4.7 KCl, 2.46 MgSO₄, 7 H₂O, and 5.56 dextrose, pH 7.4). Right ventricular afterloading was determined by relative cardiac weight ratio (blotted tissue weight, right ventricle to left ventricle plus septum) to diagnose development of PPHN (19).

Immunohistochemistry. Lung tissue was paraffin-embedded and cut in 5-μm sections, which were then deparaffinized in xylene for 20 min followed by stepwise rehydration in ethanol solutions. Formalin cross-links were removed by boiling sections in a 5 mM sodium citrate and 2 mM citric acid solution. Nonspecific antibody binding was blocked by preincubation with 10% donkey serum in Cyto-TBS+1%BSA (containing in mM: 20 Tris base, 154 NaCl, 2 EGTA, 2 MgCl₂, pH 7.4) for 20 min at room temperature in a humidified chamber. Sections were then incubated with rabbit-anti-TP-R antibody (Chemicon International) and mouse-anti-myosin heavy chain antibody (used to visualize small arteries; Abcam, Cambridge, MA) overnight at 4°C, followed by incubation with FITC-conjugated donkey anti-rabbit antibody and indocarbocyanine (Cy3)-conjugated donkey anti-mouse antibody. Sections were then mounted with antifade and visualized by fluorescent microscopy. Intensity of TP-R immunohistochemical staining was quantified in all images, employing a constant region of interest radius method, using mean intensity from a minimum of 20 regions of interest for each artery. The luminal surface of all arteries was excluded from analysis to avoid interference signal from platelets.

Cell culture. Pulmonary artery smooth muscle cells (PASMC) were obtained from newborn pigs using a dispersed cell culture method selected for myocytes (40). Third to sixth generation pulmonary arteries were obtained by microdissection into Ca²⁺-free Krebs-Henseleit physiological buffer and were allowed to recover in cold HEPES-buffered saline solution (HBS; composition in mM: 130 NaCl, 5 KCl, 1.2 MgCl₂, 1.5 CaCl₂, 10 HEPE, 10 glucose, pH 7.4) supplemented with an antibiotic/antimycotic mixture and gentamicin. Arteries were then washed twice with Ca²⁺-reduced HBS (20 μM CaCl₂) and finely minced. Arterial tissue was transferred to centrifugation at 1,200 rpm for 5 min, washed in Ca²⁺-free HBS to remove digestion solution, and then resuspended in culture medium.

The cells were plated at a density of 4.4 × 10⁴ cells/cm² in Ham’s F-12 medium with l-glutamine supplemented with 10% fetal calf serum, 1% penicillin, and 1% streptomycin. Cells were serum-deprived for 2 days once they reached confluence (in Ham’s F-12 medium with l-glutamine/penicillin/streptomycin and 1% insulin-transferrin-selenium) to synchronize cells in a contractile phenotype and then split into two groups for the final 3 days of culture: 1) control normoxic myocytes (NM), maintained serum-free in 21% O₂, 5% CO₂; and 2) hypoxic myocytes (HM), maintained serum-free in 10% O₂, 5% CO₂ for 3 days to mimic the extent and duration of the in vivo O₂ exposure.

Immunocytochemistry. PASMCs were fixed with 3% paraformaldehyde for 15 min at room temperature followed by permeabilization with 0.3% Triton X-100 for 5 min. Cells were rinsed twice with common base buffer (containing in mM: 10 MES, 150 NaCl, 5 EGTA, 5 MgCl₂, 5 glucose) and stored in Cyto-TBS at 4°C. Nonspecific binding was blocked by incubation with 10% normal donkey serum in Cyto-TBS+1%BSA for 20 min at room temperature. PASMCs were then incubated with TP-R rabbit polyclonal antibody (Cayman Chemicals, Ann Arbor, MI) overnight at 4°C, followed by incubation with FITC-conjugated donkey anti-rabbit antibody for 2 h at room temperature. Coverslips were coincubated with either mouse anti-golgii-97 (a Golgi apparatus-specific marker; Molecular Probes, Eugene, OR) or mouse anti-protein disulfide isomerase [PDI; an endoplasmic reticulum (ER)-specific marker; Stressgen Bioreagents, Victoria, British Columbia, Canada], which was followed by coincubation with Cy3-conjugated donkey anti-rabbit antibody. Nuclei were counterstained with Hoechst 33342.

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Fluorescence immunocytochemistry images were acquired using an Olympus 1X 70 microscope with an UltraPix FSI digital camera and analyzed with UltraView software (PerkinElmer).

RT-PCR. RNA was extracted from frozen PASMCs using TRIzol (Invitrogen) according to the manufacturer’s instructions. Briefly, cells were homogenized in TRIzol reagent. Chloroform was added, and samples were centrifuged at 12,000 g for 15 min at 4°C. The resulting aqueous solution was incubated in isopropyl alcohol for 10 min at 30°C. The RNA pellet was isolated by centrifuging at 8,000 g for 10 min at 4°C and washed with 75% ethanol. RNA was redissolved in diethyl pyrocarbonate-treated water at 55°C for 30 min. RNA purity was determined using a spectrophotometer. Two micrograms of total RNA was reverse transcribed using the Omniscript RT kit (Qiagen) in a total reaction volume of 20 μl in the presence of 1 μg oligo(dT)-15 primers (Promega) and 10 units of RNAse inhibitor (Promega) following the manufacturer’s instructions.

TP-R primers were used as previously published (1): sense primer 5'-TTTCCAGGCAGATTCAAC-3', antisense primer 5'-GCAGTGCTTGTCGCAACAAA-3', for an estimated product size of 200 base pairs. The reaction mixture contained 10× PCR buffer (2.5 μl), 10 mM dNTP (0.5 μl), 50 mM MgCl2 (0.75 μl), 10 μM sense primer (0.5 μl), 10 μM antisense primer (0.5 μl), platinum Taq (0.125 μl), water (18.125 μl), and 2 μl sample cDNA. PCR amplifications were carried out using a Techne Genius Unit with the following conditions: denaturation and enzyme activation at 94°C for 5 min, a total of 40 amplification cycles consisting of a 30-s denaturation at 94°C, 30-s annealing step starting at 60°C and then decreasing by 0.5°C increments per cycle until 52°C, followed by a 30-s extension at 72°C. The final extension was at 72°C for 5 min.

Pig GAPDH primers used were previously published (44): sense primer 5'-TTTCCAGGCAGATTCAAC-3', antisense primer 5'-GCAGTGCTTGTCGCAACAAA-3', for an estimated product size of 576 base pairs. The reaction mixture contained 10× PCR buffer (2.5 μl), 10 mM dNTP (1 μl), 2.5 μM sense primer (0.5 μl), 2.5 μM antisense primer (0.5 μl), water (18.125 μl), platinum Taq (0.125 μl), and 1 μl sample cDNA. PCR amplifications were carried out in the following conditions: denaturation and enzyme activation for 5 min at 95°C, 33 amplification cycles consisting of 30-s denaturation at 94°C, 30-s annealing held for 5 min, and 45-s extension phase at 72°C, with final extension held for 5 min. PCR products were separated by 1% agarose gel electrophoresis and visualized with GelStar. Bands were analyzed by densitometry, with TP-R normalized to GAPDH.

Immunoprecipitation. Whole cell lysates were collected in RIPA buffer modified for phospho-protein analysis (containing in mM: 20 MOPS, 2 EGTA, 5 EDTA, 30 sodium fluoride, 40 beta-glycerophosphate, 10 sodium pyrophosphate, 2 sodium orthovanadate, 1 PMSF, 3 benzamidine, 0.005 pepstatin A, 0.01 leupeptin). A 50% slurry of protein G Sepharose beads was prepared in lysis buffer (containing in mM: 50 Tris, 150 NaCl, 1 EDTA, 1 PMSF, and 1% Triton X-100, pH 7.4). Lysate (500 μg) was then precleared by incubation with 35 μl of 50% bead slurry in a total volume of 250 μl. Beads were isolated by centrifugation at 16,000 g for 5 min. The precleared lysate was then added to 2 μg of rabbit-TP-R antibody (Cayman Chemicals) overnight at 4°C. Thirty microliers of 50% bead slurry was then added to pull down the immunoprecipitate. Beads were washed with lysis buffer and boiled in Laemmli buffer for 10 min; the protein derived was separated by SDS-PAGE and probed with mouse-anti-phospho-serine antibody (Qiagen) and rabbit-anti-Gaα antibody (Santa Cruz Biotechnology).

To determine the signaling pathway involved in hypoxia-induced TP-R desensitization, PASMCs were incubated with 1 μM PMA (phorbol 12-myristate 13-acetate; a PKC activator) and 10 μM forskolin (a PKA activator) for the final 3 days of culture. In a separate experiment, 1 μM GTPγS (a stable GTP analog) was added to lysates during antigen-antibody incubation to maximize receptor active state conformation. TP-R phosphorylation on serine residues was then studied in all groups as described above.

Live cell calcium mobilization. Live cell calcium imaging was carried out as previously described (19). Control PASMCs or cells treated with 1 μM PMA, 10 μM forskolin, or 1 μM GTPγS (as described in Immunoprecipitation) were loaded with 5 μM fura 2-AM (Molecular Probes)/DMSO in HBSS (containing in mM: 1.26 CaCl2, 0.493 MgCl2·6 H2O, 0.407 MgSO4·7 H2O, 5.33 KCl, 0.441 KH2PO4, 4.17 NaHCO3, 137.93 NaCl, 0.338 NaHPO4, and 0.1% BSA) with 1.0 μg/ml pluronic acid as per manufacturer’s instructions. Cover glass plates were secured on an inverted microscope (Olympus) in room air and studied at ×20 magnification. Real-time ratiometric imaging of intracellular calcium concentration used excitation wavelengths of 340 and 380 nm and an emission wavelength of 510 nm; data was captured by a charge-coupled device camera and Perkin Elmer software. Each recording consisted of a stable baseline and a response to 1 μM U-46619. PMA, forskolin, and GTPγS were omitted during fura 2-AM loading but were present at the time of recording.

Statistical analysis. All data are presented as means ± SE and analyzed using an unpaired t-test with P < 0.05 considered significant.

RESULTS

Development of pulmonary hypertension was diagnosed by increased right-to-left ventricle plus septum weight ratio. Newborn piglets had a ratio of 0.709 ± 0.108 g, which decreased in control 3-day-old pigs with a ratio of 0.573 ± 0.221 g. However, this ratio was elevated in 3-day animals with hypoxic PPHN (0.877 ± 0.166 g; different from 3-day normoxic controls, P < 0.05), mainly due to an increase in right ventricular weight.

Immunohistochemical analysis of lung slices from pigs with hypoxic PPHN and age-matched controls revealed that large arteries expressed similar levels of TP-R (Fig. 1, A and B). However, in pulmonary arteries <50 μm in diameter, TP-R was not observed in control animals, whereas signal was detected in animals with hypoxic PPHN (Fig. 1C). Quantifi-
culation of TP-R intensity in the smaller caliber pulmonary arteries showed a statistically significant increase in TP-R intensity in lung slices from pigs with PPHN, with TP-R probe intensity from control arteries composed largely of background signal (Fig. 1D; \( P < 0.0001 \)).

We have previously demonstrated decreased cell surface immunostaining for TP-R and intracellular redistribution of TP-R following moderate hypoxic exposure (19). In this study, TP-R colocalization with golgin-97 (a marker for the Golgi apparatus; Fig. 2B) was not different between HM and NM. However, the relocation of TP-R in HM to the perinuclear region seemed to mirror a shift in PDI signal (a marker for the ER; Fig. 2A). Quantification of PDI signal showed that immunostaining intensity in the perinuclear region was increased in HM compared with NM (Fig. 2C; \( \text{HM} = 1.06 \pm 0.01 \text{ arbitrary units}, \text{NM} = 1.00 \pm 0.01 \text{ arbitrary units, 25 equal regions selected per microscope field image,} \ n = 10; \ P < 0.0001 \)). Total cell area containing PDI signal, normalized to number of nuclei per image, was slightly, but not significantly, decreased (Fig. 2D; \( \text{HM} = 0.91 \pm 0.03 \text{ arbitrary units,}\ \text{NM} = 1.00 \pm 0.07 \text{ arbitrary units,} \ n = 10; \ P = \text{not significant} \)).

There was no significant difference in total TP-R expression as measured by RT-PCR in HM compared with NM after normalization to GAPDH (Fig. 3).

Saturation binding experiments revealed a decrease in TP-R abundance in membrane fractions from HM compared with NM [Table 1; \( \text{NM} \text{B}_{\text{max}} \text{ (maximal binding sites)} = 610.30 \pm 270.60 \text{ fmol/mg, HM} \text{B}_{\text{max}} = 150.80 \pm 32.35 \text{ fmol/mg;} \ P < 0.0001 \)]. HM TP-R also had an increased affinity for the TP-R antagonist, SQ29548 (Table 1; \( \text{NM} \text{K}_d = 72.97 \pm 46.40 \text{nM,} \ \text{HM} \text{K}_d = 12.74 \pm 7.72 \text{nM;} \ P < 0.03 \)).

Competitive binding experiments, where unlabeled SQ29548 was used to displace \( ^3\text{H}\text{-SQ-29548} \) binding, indicated a significantly right-shifted dose-response curve in HM (Fig. 4A; \( \text{NM} \text{IC}_{50} = 1.13 \times 10^{-7} \pm 0.37 \text{ M, HM} \text{IC}_{50} = 1.17 \times 10^{-6} \pm 0.01 \text{ M;} \ P < 0.01 \)). Membrane fractions coincubated with \( ^3\text{H}\text{-SQ-29548} \) and the unlabeled TxA\(_2\) agonist U-46619 revealed a significantly left-shifted binding curve for U-46619 in HM (Fig. 4B; \( \text{NM} \text{IC}_{50} = 5.47 \times 10^{-7} \pm 0.22 \text{ M, HM} \text{IC}_{50} = 4.66 \times 10^{-10} \pm 0.18 \text{ M;} \ P < 0.005 \)).

We have previously reported that normoxic and hypoxic whole cell lysates have similar TP-R protein abundance (19). Analysis of whole cell TP-R immunoprecipitate with antibody to phosphoserine revealed that HM TP-R was significantly less phosphorylated relative to NM TP-R (Fig. 5A; \( P < 0.003; \ n = 5 \)). Conversely, immunoblot of the TP-R immunoprecipitate with a G\(_{\text{q}\alpha}\) antibody indicated a greater association of HM TP-R with G\(_{\text{q}\alpha}\) than was the case for NM TP-R (Fig. 5B; \( P < 0.03; \ n = 3 \)). The immunoprecipitates contained similar amounts of TP-R (Fig. 5C; \( P = \text{not significant}; \ n = 4 \)).

The elevated phosphorylation state of the NM TP-R compared with HM TP-R was maintained following immunoprecipitation in the presence of GTP\(_\gamma\)S, used to ensure maximal receptor activation (Fig. 6, A and B; \( P < 0.03; \ n = 3 \)). Incubation with PMA (a PKC activator) increased TP-R serine phosphorylation in the hypoxic group alone (Fig. 6, A and B; \( P < 0.04; \ n = 3 \)). However, following incubation with forskolin (a PKA activator), there was a significantly higher level
of phosphorylation of both NM and HM TP-R, ablating any difference in receptor phosphorylation between the two groups (P = not significant; n = 3).

As previously observed, PASMC exposed to 10% O2 for the final 3 days of culture resulted in an elevated peak [Ca2+]i response to 1 μM U-46619 (19). Incubation of both HM and NM with GTPγS had no effect on peak calcium response to 1 μM U-46619, and the hypoxia-induced elevation in agonist response was maintained (Fig. 6C; NM 1.088 ± 0.224 μM, HM 2.142 ± 0.155 μM, n = 16; P < 0.01). The hyperresponsiveness of the HM was also maintained following incubation with PMA (Fig. 6C; NM 1.177 ± 0.185 μM, HM 2.253 ± 0.228 μM, n = 16; P < 0.01). In contrast, forskolin markedly inhibited the hypoxia-induced increase in peak calcium response to 1 μM U-46619 (Fig. 6C; NM 0.513 ± 0.099 μM, HM 0.630 ± 0.056 μM; P = not significant).

DISCUSSION

In this study, we examined the effect of moderate hypoxia on TP-R localization and kinetics in a neonatal PASMC culture model of hypoxic pulmonary hypertension to determine the mechanism by which these cells become hypersensitive to a TxA2 agonist. We determined that TP-R localization is altered response to 1 μM U-46619 (19). Incubation of both HM and NM with GTPγS had no effect on peak calcium response to 1 μM U-46619, and the hypoxia-induced elevation in agonist response was maintained (Fig. 6C; NM 1.088 ± 0.224 μM, HM 2.142 ± 0.155 μM, n = 16; P < 0.01). The hyperresponsiveness of the HM was also maintained following incubation with PMA (Fig. 6C; NM 1.177 ± 0.185 μM, HM 2.253 ± 0.228 μM, n = 16; P < 0.01). In contrast, forskolin markedly inhibited the hypoxia-induced increase in peak calcium response to 1 μM U-46619 (Fig. 6C; NM 0.513 ± 0.099 μM, HM 0.630 ± 0.056 μM; P = not significant).

Table 1. Thromboxane receptor saturation binding kinetics

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<th>Kd (nM)</th>
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<tr>
<td>NM</td>
<td>72.97±46.40</td>
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<td>HM</td>
<td>12.74±7.72*</td>
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Values are represented as means ± SE. *P < 0.03; **P < 0.002. Bmax, maximal binding sites; NM, normoxic myocytes; HM, hypoxic myocytes.
both in an in vivo model of hypoxia-induced PPHN, as well as in pulmonary artery myocytes exposed to moderate hypoxia in vitro. After whole animal hypoxia, TP-R appeared in smaller caliber pulmonary arteries where normally TP-R is not expressed. In HM, there was an intracellular shift in TP-R distribution that followed a change in ER distribution. We also observed increased TP-R binding affinity, decreased receptor phosphorylation of serine residues, and an increased coupling to the G\( \alpha_q \) protein in pulmonary arterial smooth muscle following hypoxic exposure. Lastly, we found that forskolin treatment increased TP-R phosphorylation in HM and reduced agonist-induced peak [Ca\(^{2+} \)]\( _i \) response back to control levels.

In discussing these data, we acknowledge certain limitations in application of the in vitro hypoxia model to the pathophys-

![Graph A](image1)

![Graph B](image2)

![Graph C](image3)

Fig. 4. Competitive binding kinetics: 100 \( \mu \)g of PASMC membrane was incubated with 10 nM \(^3\)H-SQ-29548 and a range of concentrations of unlabeled TP-R antagonist (SQ29548; A) or unlabeled TP-R agonist (U-46619; B). The IC\(_{50}\) values for HM and NM were significantly different in both cases (A, \( P < 0.01 \); B, \( P < 0.005 \)). Data were obtained from cell lysates from 13 animals, and experiments were performed 3 times.

![Graph D](image4)

Fig. 5. Thromboxane receptor coimmunoprecipitation: the immunoprecipitate obtained by HM and NM whole cell lysate incubation with a TP-R antibody was probed with mouse-anti-phosphoserine (P-serine) antibody (A) or rabbit-anti-G\( \alpha_q \) antibody (B). HM had significantly less phosphorylation of TP-R than NM (A; \(* P < 0.003 \); \( n = 5 \)), whereas HM TP-R was associated significantly more with G\( \alpha_q \) protein (B; \#\( P < 0.03 \); \( n = 3 \)). C: the relative amount of TP-R precipitated by whole lysate incubation was not significantly different between NM and HM (\( P = \) not significant; \( n = 4 \)).
Altered TP-R expression. An increase in TxA2 production, in conjunction with an increase in COX-2 abundance, has been described in the piglet model of hypoxia-induced PPHN (38). Others have reported an increase in TxA2 following septic induction of PPHN in the piglet (13). Additionally, TP-R activation, although not through action of TxA2, has been shown to be involved in the increase in endothelin-1 in the rat model of neonatal PPHN (22). Altered receptor abundance for TxA2 has not been previously characterized in hypoxia. In this study, we observed an increase in TP-R expression in small caliber pulmonary arteries. The histology of end-stage pulmonary hypertension is well characterized by thickened vascular media and adventitia, hyperplasia and hypertrophy of the vascular smooth muscle layer, and increased extracellular matrix deposition (30), impairing vascular dispensability (45) and eventually resulting in a fixed and irreversible increase in pulmonary vascular resistance. In vivo hypoxia clearly also results in distal propagation of TP-R expression, such that 10–50-μm diameter arteries accrue receptors capable of responding to circulating TxA2. Thus, whereas under normal conditions, only pulmonary arteries 100 μm in diameter or larger may be responsible for TxA2 agonist-induced vasoconstriction, after development of PPHN, smaller arteries become capable of contributing to agonist response and overall PA pressure. It should be noted that this immunohistochemistry data indicates whole cell TP-R expression, not cell surface expression, and the latter decreases under in vitro hypoxia. Both control and hypoxic cultured pulmonary artery myocytes expressed TP-R, as indicated by RT-PCR. The pulmonary arteries microdissected for the primary culture preparation contained a mixture of larger and smaller vessels (3rd to 6th generation intrapulmonary branches). As smaller vessels have greater muscular content, the majority of cultured myocytes derive from the smaller arteries. Both control and hypoxic cultured pulmonary artery myocytes abundantly expressed TP-R, as shown by RT-PCR. Expression of TP-R in normoxia may have been simply induced under cell culture conditions, or may reflect contribution from smooth muscle cells from larger arteries, as vessel size had an important impact on TP-R expression in our study. Therefore, a limitation in interpretation of this study is that altered regulation of TP-R observed in cultured myocytes may not be entirely representative of TP-R alterations in vivo, depending on the original location of the studied TP-R within the arterial tree.

TP-R intracellular localization. We have previously shown that moderate hypoxic exposure alters TP-R localization following in vitro hypoxia for 3 days, with decreased cell surface expression and a translocation of the intracellular receptor to the perinuclear region (19). Cell surface receptor abundance is known to be modulated by internalization and ER-associated degradation (47). Agonist-induced internalization of the TP receptor is mediated by Goq signaling (38). In the context of an increased TxA2:PGI2 milieu and increased receptor affinity, TP-R internalization in hypoxia may constitute a negative feedback mechanism attenuating vasoconstrictor response. In this study, we also observed that the ER relocates to a perinuclear position in HM, although this change was numerically small. There is precedent for this observation: a reduction in smooth ER has been described in rat hepatocytes following exposure to 5% environmental O2 (55). The admittedly minor (albeit statistically significant) change in the ER morphology of...
HM may explain the observed shift in intracellular TP-R distribution, which may impact on receptor internalization and cycling. Observations reported elsewhere of altered TP-R localization following oxidative stress, involving stabilization of the TP-R and translocation from the ER to the Golgi apparatus following exposure to H2O2 (46), describe a change in cell surface receptor immunostaining within this range. A three-dimensional analysis of ER distribution ascribes functional specialization of protein import machinery to ER lamellae organized a few nanometers apart (11), suggesting small differences in ER distribution may confer significant changes of function. Luminal ER protein chaperones such as PDI (used in this study as an ER marker) function as protein escorts, hence minor alterations in localization of this protein may have functional significance (26, 31). The slight shift in ER distribution we observe in HM may alter receptor cycling, posttranscriptional modification, and/or compartmentalization, which would impact on cell surface abundance and activity of the TP-R, as we report is the case in HM.

**TP-R binding kinetics.** Published values of SQ29548 binding to TP-R range from a $K_d$ of 6.3 nM (46) to 1.72 nM (16). The $K_d$ value we obtained for HM was comparable to previously reported values, whereas the NM $K_d$ was relatively elevated, suggesting that the NM TP-R is relatively desensitized. The $B_{max}$ was also decreased in HM TP-R compared with NM, supporting our previous observation by immunocytochemistry (19). After observing an alteration in both TP-R abundance and binding affinity following hypoxic exposure, it was necessary to examine competitive binding kinetics. As the desired receptor-ligand interaction involved the agonist rather than the available radiolabeled antagonist, the competitive binding analysis was carried out against both unlabeled SQ29548 (a TP-R antagonist) and unlabeled U-46619 (a TP-R agonist). Published values of SQ29548 binding to TP-R range from a $K_d$ of 6.3 nM (47) to 1.72 nM (16). In NM, unlabeled SQ29548 had a lower IC$_{50}$, suggesting increased affinity for antagonist in the normal condition compared with hypoxic cells, a finding inconsistent with that observed in saturation binding experiments; this may have been an artifact of the greater cell surface receptor abundance ($B_{max}$) in NM, leading to increased availability of open receptor for binding. However, unlabeled U-46619 displaced the ³H-SQ-29548 in HM at significantly lower concentrations, suggesting that the hypoxic receptor has increased affinity for the agonist; this observation supports the saturation binding kinetics. The difference in agonist/antagonist displacement of the labeled antagonist may be due to the consistently lower $K_d$ of SQ29548 compared with the $K_d$ for U-46619 (29). Since it is only activation of the TP-R by U-46619 that leads to increased [Ca$^{2+}$]$_i$ and subsequent smooth muscle contraction, the significance of the competitive binding data lies in the clear indication that the hypoxic TP-R has an increased affinity for the agonist.

**TP-R phosphorylation.** The two known isoforms of mammalian TP receptor share the first 328 residues but differ at the COOH-terminal end (34); TP isoform may determine specificity of interaction with Go protein subunits, but both couple to PLC similarly, and no major differences in ligand affinity have been identified (2). Both Txa2 receptor isoforms are regulated by COOH-terminal serine phosphorylation (12). TPx is phosphorylated and desensitized by pulmonary circuit relaxants (37). Upon agonist-induced COOH-terminal phosphorylation, the TPβ isoform is amenable to β-arrestin binding (35), which results in desensitization, uncoupling from heterotrimeric G protein, and actin-dependent receptor endocytosis (25). When oligomerized with TPβ, TPα will also undergo endocytosis (24). In this study, phosphorylation state of the normoxic TP-R was elevated compared with hypoxic TP-R. The hypoxic TP-R had increased association with Go$_{q}$ compared with normoxic TP-R, suggesting that downstream contractile signaling in hypoxic TP-R may be augmented compared with the relatively desensitized normoxic TP-R. As this dephosphorylation of hypoxic TP-R occurred in the context of increased receptor internalization, we speculate that TP-R internalization may represent receptor cycling as a consequence of increased Txa2 production, receptor sensitization, and/or downstream signaling in hypoxia, whereas decreased TP-R phosphorylation state may be mediated by the contrary loss of vasorelaxant stimulation in HM.

**Regulation of TP-R phosphorylation.** Covalent modification of various GPCRs has been shown to regulate their activity due to alterations in active state conformation or to regulatory phosphorylation. Activity of smooth muscle TP-R is primarily regulated by serine phosphorylation. Ser331 on the COOH-terminal TP-R tail is known to be phosphorylated by PKC, resulting in desensitization (57). There is evidence that TPα, but not TPβ, may be subject to cross-desensitization mediated by prostaglandin D$_2$ receptor (DP), and occurring via direct PKA-mediated phosphorylation of TPα at Ser329 after DP stimulation (10). Signaling by TPα, but not TPβ, is also subject to PGI2-induced desensitization (via IP-prostanoid receptor stimulation) mediated by PKA phosphorylation of Ser329 (51). An independent mechanism of TP-R desensitization involves direct PKG phosphorylation of Ser331 in response to NO (37). Hypoxia causes sensitization of the TP-R in neonatal pulmonary artery myocytes, but under control conditions, the TP-R is relatively desensitized due to regulatory phosphorylation. TP-R phosphorylation and peak [Ca$^{2+}$]$_i$ response to agonist is unaffected by GTP-induced increase in active state conformation, as maximal activation of the TP-R had no effect on phosphorylation state of the receptor, and peak [Ca$^{2+}$]$_i$ response to Txa2 agonist remained significantly elevated in HM. PKC activation increases HM TP-R phosphorylation; however, the [Ca$^{2+}$]$_i$ response to U-46619 remained elevated compared with normoxic controls, suggesting that in neonatal pulmonary artery myocytes, PKC may target residues with no direct effect on receptor-induced Ca$^{2+}$ signaling. Incubation with a direct activator of PKA resulted in markedly increased TP-R phosphorylation, ablatting the difference between hypoxia and normoxia. PKA activation also inhibited the hypoxia-induced increase in peak [Ca$^{2+}$]$_i$ response to U-46619, suggesting that PKA-targeted serine residues on TP-R are involved in normoxic desensitization of TP-R.

Protein phosphatases PP1 and PP2A are implicated in TP-R dephosphorylation (42). We have previously reported a decrease in PP1M (myosin phosphatase) activity in hypoxic neonatal pulmonary artery; PP2 activity was not altered (3). This has also been reported in hypoxic PA myocytes (53). The mechanism by which TP-R is dephosphorylated in hypoxia falls outside the scope of this paper but deserves further study.

We conclude that hypoxia in the perinatal pulmonary circuit causes: distal propagation of Txa2 receptor expression; increased TP-R agonist affinity despite a decrease in $B_{max}$ re-

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resulting from increased receptor internalization; and a loss of basal TP-R phosphorylation, resulting in increased coupling of the receptor complex to vasoconstrictor signaling intermediates. We speculate that, under normal conditions in the pulmonary circuit, the neonatal TP-R is relatively desensitized compared with the adult TP-R due to increased serine residue phosphorylation. This may be physiologically advantageous, as pulmonary arteries would be less able to constrict in response to circulating TxA2 and therefore would not hinder normal circulatory transition. However, after exposure to hypoxia, TP-R appears in smaller pulmonary arteries and becomes dephosphorylated, which increases its affinity for TxA2 agonist U-46619 and increases coupling to Gαq. Regulatory phosphorylation of TP-R in the neonatal pulmonary circuit may be mediated via the PKA pathway; PKA activation results in TP-R phosphorylation and can inhibit development of hypoxia-induced TP-R hypersensitivity. Altered TP-R localization and kinetics in HM may result in inflammatory agonist hypersensitivity in resistance level pulmonary arteries, which would contribute to the increased pulmonary arterial pressure observed in PPHN and could interfere with current PPHN therapies.

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